EFFECTS OF VEGETABLE TANNINS ON GLUCOSE OXIDATION BY VARIOUS MICROORGANISMS

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Abstract

The effects of purified tannin preparations of chestnut wood and of wattle bark on the respiration of resting cells of microorganisms were measured in a Warburg apparatus. Tannins were tested at 0.5% (w/v) concentration alone and in mixtures with glucose which provided energy for the microorganisms. In presence of the chestnut and wattle tannins, the exogenous respiration of *Azotobacter vinelandii* was reduced by 50 and 85% and that of *Escherichia coli* by 40 and 20%, respectively; respiration of *Azotobacter chroococcum* was completely inhibited by either tannin. Glucose oxidation by *Rhizobium meliloti*, *Rhizobium* sp., and *Saccharomyces cerevisiae* was inhibited by tannins to small degrees. Tannins had no effect on glucose utilization by *Rhodotorula* sp. and *Pseudomonas fluorescens*. *A. vinelandii*, *E. coli*, and *P. fluorescens* utilized tannins, especially wattle, as an energy source.

Introduction

By definition, tannins are inorganic or organic substances capable of converting easily putrescible animal skins to a stable product, leather (13, 34). Plants produce a variety of highly reactive polyhydroxy phenols that are extractable with water to yield commercial tannins (18, 19, 23, 34). Such substances occur mainly among higher plants, but chemically similar materials are also found in tissues of lower plants like ferns, mosses, liverworts, algae, or fungi (18). In higher plants, tannins are produced in roots, stems, bark, wood, and leaves, often constituting 5 to 15% or more of the dry weight of such tissues (18, 19, 23, 36).

In forests, precipitation removes considerable amounts of polyhydroxy phenols from the dead wood, bark, or fallen leaves and needles, and leaches these substances into the soil. Here, with time, the deposited phenols are partially or completely decomposed by various microorganisms. Little is known of the effects of tanning substances on the physiological activities of soil microorganisms. This study concerns the effects of purified tannin preparations on the respiration of various microorganisms, mostly from soil, by the Warburg manometric method.

Materials and Methods

Tannin Preparations

Tannin materials used in this study were chestnut wood and a mimosa or wattle bark. A little oak wood was mixed with the chestnut but constituted less than 1% of the mixture. Polyhydroxy phenolic substances of the chestnut wood (Fagaceae) represented the "hydrolyzable tannins". In this group of tannins, gallic acid or related aromatic acids are ester-linked to a central carbohydrate core. Tannin molecules of this type are readily broken down by

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TABLE I

Analyses of the purified tannin preparations

Source	pH, aqueous solution 0.5% (w/v)	Tannin, %	Non-tannin, %	In-solubles, %	Moisture, %	Lead, p.p.m.
Chestnut wood	3.7	93.6	1.2	0	5.2	102
Wattle bark	3.9	92.8	1.7	0	5.5	256

hydrolysis with acids, alkalies, or enzymes (34). Polyhydroxy phenolic substances of the wattle bark (Leguminosae) represented the "non-hydrolyzable or condensed tannins". In this second group, the phenolic nuclei are connected by carbon-to-carbon links and are not chemically bound to a carbohydrate (34). Usually the tannin extracts of any plant contain substances of both groups, but generally those of one group predominate (34).

Before respirometric measurements were made, the tannin materials of the chestnut and wattle were purified by removing sugars, hemicelluloses, and other non-tannins which would obscure the effects of the real tannins (3). This purification was accomplished by the lead acetate method of Roux (28). The tannin and lead contents of the purified preparations were determined by a hide powder method (2) and dithiozone procedure (30) respectively.

The purified tannin preparations were amorphous, powdery when ground, and readily soluble in water. The material derived from chestnut was light orange and that from wattle was brick-red in color. Analyses of the materials are shown in Table I.

The purified tannins were dried in an oven at 60 °C for about 2 hours to remove the excess moisture and residual ethyl alcohol which might not have been removed completely by vacuum drying during the purification procedure. After they were dried, the materials were stored in stoppered jars.

Approximately 20 to 24 hours before the respirometric measurements were begun, 163-mg amounts of purified tannin preparations were dissolved in 25-ml amounts of $M/15$ phosphate buffer of pH 5.8 or 6.8. Phenolic tannins, being acid (Table I), lowered the pH of the original buffer solutions by as much as 1.5 units. The reaction of these buffered solutions of tannins was adjusted with the aid of a pH meter to the optimum for the microorganisms to be tested by the addition of very small amounts of 0.1 or 1.0 N NaOH. After pH adjustment the tannin concentration in the buffer solutions was about 0.65% (w/v).

Preliminary manometric measurements showed that the phenolic substances of freshly dissolved tannins in a $M/15$ phosphate buffer underwent autoxidation, consuming greater amounts of atmospheric oxygen than did the azotobacter or yeast cell suspensions oxidizing glucose in the presence of tannins. Therefore on the day before manometric determinations were made, the tannin solutions at the desired pH were exposed to atmospheric oxygen at room temperature for about 4 hours with intermittent shaking to stabilize the phenolic substances with respect to oxidation. The solutions were then refrigerated until use. This treatment caused the chestnut tannin solutions to become dark green in color and those of wattle, dark brown.

Microorganisms

The eight microorganisms used in the study and their sources were *Azotobacter vinelandii* and *Azotobacter chroococcum* (Dr. P. W. Wilson, Department of Bacteriology, University of Wisconsin, Madison); *Rhizobium meliloti*, *Escherichia coli*, and *Pseudomonas fluorescens* (Department of Microbiology, University of Guelph, Guelph, Ontario); *Saccharomyces cerevisiae* (Dr. R. Bandoni, Department of Biology and Botany, University of British Columbia, Vancouver); and *Rhizobium* sp. and a red yeast, *Rhodotorula* sp. isolated by the writer from a root nodule of vetch and from fallen oak leaves, respectively.

The microorganisms were propagated at 28 °C in 250-ml quantities of liquid media in 1-liter flasks. Azotobacter organisms were grown for 7 days in N-free medium 77 of Allen (1) in which mannitol was replaced by an equal amount of glucose and to which 1.0 mg of sodium molybdate was added per liter of solution. The rhizobia were grown for 5 days in the yeast–extract–mannitol medium 77 (1) in which 1.0 g per liter of yeast extract (Difco) was used as a source of nitrogen. *E. coli* and *P. fluorescens* were cultivated in nutrient broth (Difco) for 3 days. *S. cerevisiae* and *Rhodotorula* sp. were grown in Czapek's medium for 5 and 8 days respectively. None of the cultures was grown in the presence of tannins before respirometric measurements were made.

For respirometry, the microbial cultures were sedimented by centrifugation, and washed 3 times with cold distilled water. The cells were resuspended in 25-ml portions of $M/15$ phosphate buffer and they were used either immediately or after 1 or 2 hours of storage at 5 °C. The nitrogen content of each suspension was determined in duplicate by a semimicroKjeldahl procedure. However, because comparisons between species were not to be made, it was desirable to present data as oxygen uptake per flask rather than on a nitrogen basis.

Measurements of Microbial Respiration

Respiration was measured by the direct method according to Umbreit *et al.* (33). Reaction vessels each contained 3.25 ml of liquids. A 2.5-ml aliquot of the appropriate tannin solutions, chestnut or wattle (0.65% w/v), and 0.05 ml of glucose solution (1.0 or 2.0 *M*) were dispensed into the main chamber of some vessels. For controls, 2.5 ml phosphate buffer replaced the tannin solution and 0.05 ml distilled water replaced the glucose solution. The cell suspension, 0.5 ml per vessel, was placed in the sidearm. The center well contained 0.2 ml of a 20% KOH solution. As a result of the dilution, the final tannin concentration in Warburg vessels was approximately 0.5% (w/v). Treatments that included glucose were tested in triplicate; the remaining treatments were in duplicate, except that those which included only a tannin with cells but no glucose were tested singly. The flasks were arranged randomly on a Warburg apparatus operating at 80 strokes per minute. The water bath temperature was 30 °C for tests with *Azotobacter* and *Rhizobium* species; 25 °C with *P. fluorescens*, *S. cerevisiae*, and *Rhodotorula* sp.; and 37 °C with *E. coli*.

At the end of each experiment the liquid in the main chamber of each vessel was removed with a capillary pipette and its pH determined electrometrically. Occasionally, cells in some vessels were examined microscopically. No differ-

ences in cell morphology were observed, but those cells exposed to tannins appeared to be very slightly coated with tannins.

Results

The effect of chestnut and wattle tannins on glucose oxidation by eight microorganisms is illustrated in Figs. 1 and 2. The data represent the exogenous respiration of the microorganisms utilizing glucose, glucose plus tannins, and tannins without glucose; the endogenous respiration and oxygen uptake caused by oxidation of tannins in the absence of cells have been subtracted from the appropriate values of exogenous respiration.

A. chroococcum was the most sensitive to tannins of the eight species tested (Figs. 1 and 2). The calculated values for exogenous oxygen consumption were negative, thus indicating that glucose oxidation by this organism was

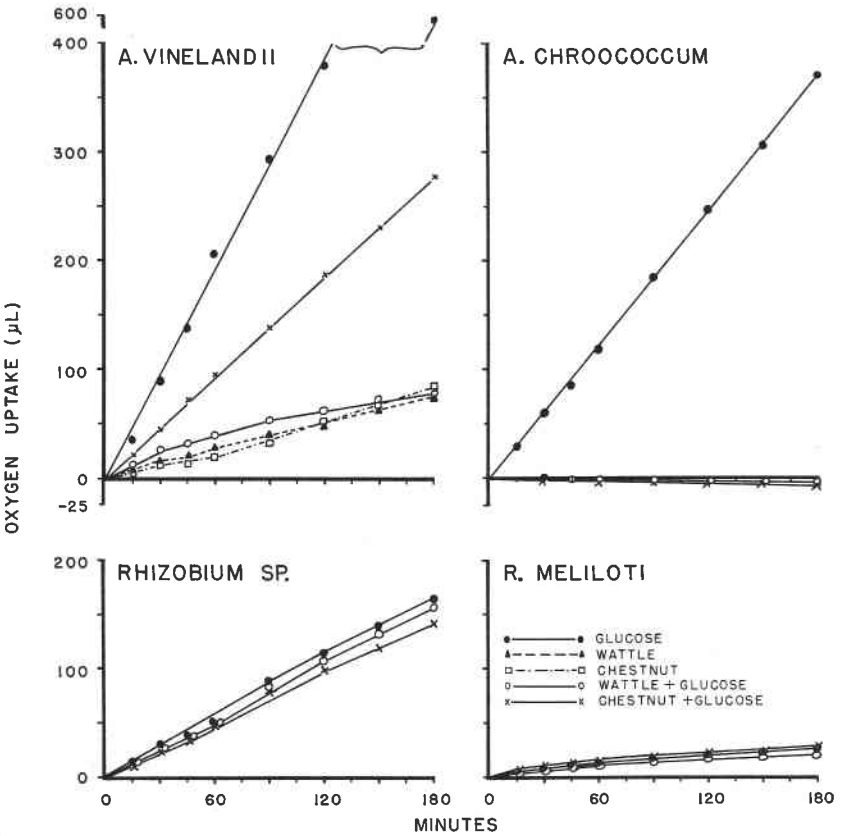

FIG. 1. Oxidation of glucose and tannins by *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Rhizobium* sp., and *Rhizobium meliloti*. Vessels with cells of *A. vinelandii* and *A. chroococcum* contained 1.06 and 1.23 mg of cell N respectively and 100 µmoles of glucose per vessel; the initial reaction of the solutions was pH 6.60. Vessels with *Rhizobium* sp. and *R. meliloti* contained 0.54 and 0.73 mg of cell N respectively and 50 µmoles of glucose per vessel; the initial reaction of the solutions was pH 6.80.

completely inhibited by tannins. Percentage reductions in oxygen consumption during glucose oxidation by the organisms in the presence of wattle and chestnut tannins in the 3-hour test period were, respectively, *A. chroococcum*, 100 and 100; *A. vinelandii*, 85 and 50; *Rhizobium* sp., 6 and 14; *R. meliloti*, 36 and 0; *E. coli*, 20 and 40; *P. fluorescens*, 0 and 0; *S. cerevisiae*, 10 and 16; and *Rhodotorula* sp. 6 and 7 (Figs. 1 and 2). It is noteworthy that the tannins did not inhibit glucose oxidation by *P. fluorescens*; on the contrary, this organism used the phenolic tannins, especially wattle, taking up 15% more oxygen when incubated in wattle tannin than in glucose alone. Also in the presence of chestnut and wattle tannin, without glucose, *Pseudomonas fluorescens* consumed, respectively, 46 and 70% of the amount of oxygen that it took up during the assimilation of glucose (Fig. 2).

Examination of contents of the flasks after 3 hours of incubation showed that the suspensions were adequately buffered. The maximum reductions in

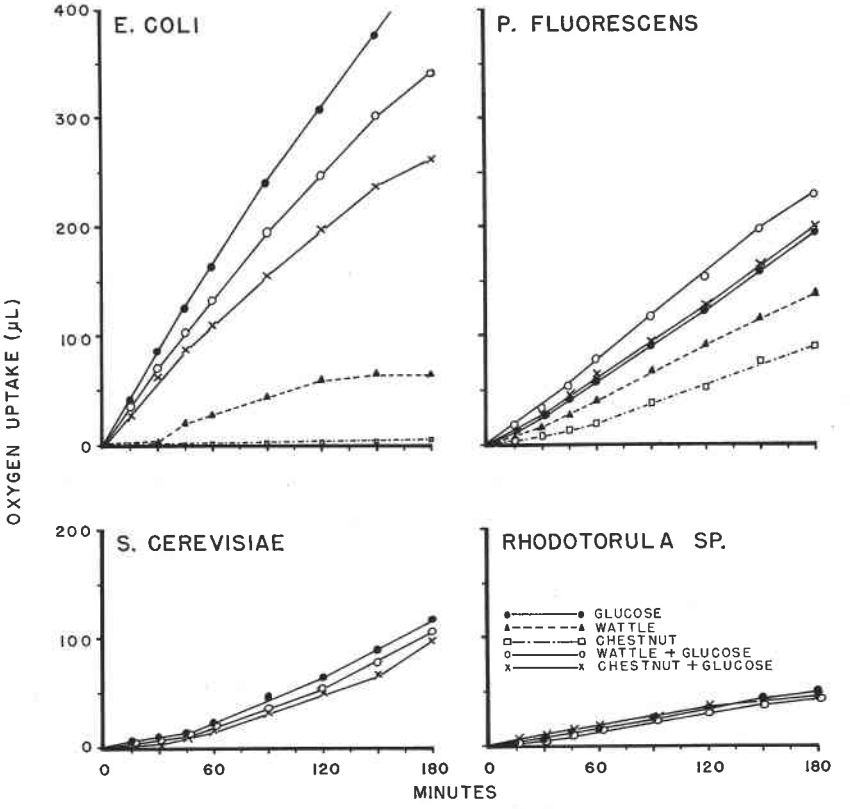

FIG. 2. Oxidation of glucose and tannins by *Escherichia coli*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, and *Rhodotorula* sp. Vessels with cells of *E. coli* and *P. fluorescens* contained 0.68 and 0.74 mg of cell N respectively and 50 µmoles of glucose per vessel; the initial reaction of the solutions was pH 6.50. Vessels with *S. cerevisiae* and *Rhodotorula* sp. contained 0.58 and 0.40 mg of cell N respectively and 100 µmoles of glucose per vessel; the initial reaction of the solutions was pH 5.40.

pH in this period were 0.05 by both azotobacters; 0.01 and 0.40 by *R. meliloti* and *Rhizobium* sp. respectively; 0.05 by *P. fluorescens* and *E. coli;* 0.10 by *Rhodotorula* sp.; and 0.80 by *S. cerevisiae*. The greatest changes in pH values occurred mainly in systems made of buffer, glucose, and cell suspensions. These changes in pH and the concentration of lead (1 p.p.m., as estimated from the data of Table I) in the flask contents were believed to exert very little, if any, inhibitory effect on respiration.

Discussion

This study revealed that azotobacter organisms were more sensitive to 0.5% tannin concentration than the other microorganisms tested. Wattle tannin inhibited respiration of azotobacters to a greater extent than did the chestnut tannin. Wattle tannin suppressed growth of sulfur-reducing bacteria (6) and decreased nitrate production in soil (4) to a greater extent than did the chestnut tannin. Certain organic substances, probably polyhydroxy phenols, derived from plant materials were claimed to inhibit growth of azotobacter in soil (29). It has also been reported (8) that metabolic activities of azotobacter organisms may be stimulated or inhibited by the phenolic extracts of plant tissues.

This study did not suggest how the polyhydroxy phenols (tannins) interfered with the oxygen uptake of the microorganisms. Roux (27) and the writer (unpublished results) showed that tannins are readily oxidized by atmospheric oxygen and that the oxidation rates increase markedly as the reaction of the medium is changed from a strongly acid to a strongly alkaline pH. It is unlikely, however, that the tannins were competing with respiring cells for the availability of oxygen in the present tests. The respiration rates of *P. fluorescens* and *Rhodotorula* sp., for example, were not reduced by the presence of tannins in solutions. Also, the systems containing microbial cells and tannins were well aerated in the Warburg respirometer.

Reductions in the oxygen uptake by the microorganisms that utilized glucose in the presence of tannins were probably brought about by a combination of factors. Tannins could have reacted with the protein of microbial cells in a manner similar to their reaction with the collagen of hide in the production of leather. Consequently, the coating of cells may have affected the uptake of substrate by the microbial cells. The reaction of vegetable tannins with collagen of skins has been attributed to such effects as hydrogen bonding, electrostatic bonding, van der Waal and London's forces, and a physical deposition of tannins on the protein (13, 14, 22, 24, 34, 35). Furthermore, there are reports that vegetable tannins are capable of inactivating plant viruses (32), of protecting certain plants from fungal diseases (21), or of inactivating enzymes by precipitation (12, 15). Also, plant polyphenols are known to be able to chelate iron, copper, calcium, zinc, aluminium, and possibly some other inorganic elements (7, 11, 16, 17, 20, 31).

P. fluorescens, *E. coli*, and *A. vinelandii* appeared to utilize the tannins as a carbon source. In a subsequent study by the author (unpublished results) *P. fluorescens* and *E. coli* were grown for 13 months in a mineral solution containing 0.2% wattle tannin as the only source of carbon; *A. vinelandii* could fix small amounts of atmospheric nitrogen in 0.1% tannin solutions using the

carbon of tannins for growth. Other workers (5, 9, 10, 25, 26) have demonstrated the ability of pseudomonads to break down plant polyphenols, hydrocarbons, and other aromatic compounds.

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